P-6149

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re U.S. Patent Application		)
Applicant:	Botha et al.	)
Serial No.:	09/779,237	)
Filed:	February 8, 2001	)
For:	The Regulation and Manipulation of Sucrose Content in Sugarcane	)

CERTIFIED COPY OF PRIORITY APPLICATION

Sertifikaat

REPUBLIEK VAN SUID-AFRIKA

Certificate

PATENTKANTOOR

PATENT OFFICE

DEPARTEMENT VAN HANDEL EN NYWERHEID

REPUBLIC OF SOUTH AFRICA

DEPARTMENT OF TRADE AND INDUSTRY

Hiermee word gesertifiseer dat
This is to certify that the documents attached hereto are true copies of the Forms P2, P6, provisional specification and drawings of South African Patent Application No. 2000/0574 in the name of South African Sugar Association

Filed : 8 February 2000

Entitled : The Regulation and Manipulation of Sucrose Content in Sugarcane

Geteken te PRETORIA in die Republiek van Suid-Afrika, hierdie dag van
Signed at PRETORIA in the Republic of South Africa, this day of

Registrateur van Patente
Registrar of Patents

FORM P.2

REPUBLIC OF SOUTH AFRICA | REGISTER OF PATENTS | PATENTS ACT, 1

OFFICIAL APPLICATION	LODGING DATE: PROVISIONAL	ACCEPTANCE DATE
21 01 20000574	22 8 FEBRUARY 2000	47
INTERNATIONAL CLASSIFICATION	**LODGING DATE: COMPLETE**	**GRANTED DATE**
51	23	

FULL NAME(S) OF APPLICANT(S)/PATENTEE(S)

71 SOUTH AFRICAN SUGAR ASSOCIATION

APPLICANTS SUBSTITUTED:	DATE REGISTERED
71	

ASSIGNEE(S)	DATE REGISTERED
71	

FULL NAME(S) OF INVENTOR(S)

72
1. BOTHA; FREDERIK COENRAAD
2. GROENEWALD; JAN HENDRIK

PRIORITY CLAIMED	COUNTRY	NUMBER	DATE
N.B. Use International abbreviation for country (see Schedule 4)	33 NONE	31 NONE	32 NONE

TITLE OF INVENTION

54 THE REGULATION AND MANIPULATION OF SUCROSE CONTENT IN SUGARCANE

ADDRESS OF APPLICANT(S)/PATENTEE(S)

MOUNT EDGECOMBE, KWAZULU-NATAL, SOUTH AFRICA

ADDRESS FOR SERVICE	S AND F REF
74 **SPOOR AND FISHER, SANDTON**	JP/T 551 /al

PATENT OF ADDITION NO.	DATE OF ANY CHANGE
61	
FRESH APPLICATION BASED ON	**DATE OF ANY CHANGE**

SPOOR AND FISHER

2000/00574

FORM P.1

REPUBLIC OF SOUTH AFRICA
PATENTS ACT, 1978
APPLICATION FOR A PATENT
AND ACKNOWLEDGEMENT OF RECEIPT
(Section 30 (1) – Regulation 22)

REVENUE
R 60,00
REPUBLIEK VAN SUID AFRIKA

The granting of a patent is hereby requested by the undermentioned applicant on the basis of the present application filed in duplicate

OFFICIAL APPLICATION NO.			S AND F REFERENCE
21	01	20000574	JP/T 551 /al

FULL NAME(S) OF APPLICANT(S)

71	SOUTH AFRICAN SUGAR ASSOCIATION

ADDRESS(ES) OF APPLICANT(S)

	MOUNT EDGECOMBE, KWAZULU-NATAL, SOUTH AFRICA

2000 -02- 08

TITLE OF INVENTION

54	THE REGULATION AND MANIPULATION OF SUCROSE CONTENT IN SUGARCANE

THE APPLICANT CLAIMS PRIORITY AS SET OUT ON THE ACCOMPANYING FORM P.2 THE EARLIEST PRIORITY CLAIM IS:

COUNTRY:	NUMBER:	DATE:
NONE	NONE	NONE

~~THIS APPLICATION IS FOR A PATENT OF ADDITION TO PATENT APPLICATION NO~~.

21	01	

~~THIS APPLICATION IS A FRESH APPLICATION IN TERMS OF SECTION 37 AND IS BASED ON APPLICATION NO~~.

21	01	

THIS APPLICATION IS ACCOMPANIED BY:

- [x] 1. A single copy of a provisional ~~or two copies of a complete~~ specification of 8 pages.
- [x] 2. Drawings of 4 sheets.
- [ ] 3. Publication particulars and abstract (Form P.8 in duplicate).
- [ ] 4. A copy of Figure ..... of the drawings (if any) for the abstract.
- [ ] 5. An assignment of invention.
- [ ] 6. Certified priority document(s) .
- [ ] 7. Translation of the priority document(s).
- [ ] 8. An assignment of priority rights.
- [ ] 9. A copy of the Form P.2 and the specification of S.A. Patent Application No.
- [ ] 10. A declaration and power of attorney on Form P.3.
- [ ] 11. Request for ante-dating on Form P.4.
- [ ] 12. Request for classification on Form P.9.
- [x] 13. Form P.2 in duplicate.

74	ADDRESS FOR SERVICE: SPOOR AND FISHER, SANDTON

Dated: 8 FEBRUARY 2000

SPOOR AND FISHER
PATENT ATTORNEYS FOR THE APPLICANT(S)

RECEIVED

REGISTRAR OF PATENTS

REPUBLIC OF SOUTH AFRICA
PATENTS ACT, 1978

PROVISIONAL SPECIFICATION

(Section 30(1) – Regulation 27)

OFFICIAL APPLICATION NO.		LODGING DATE	
21 01	20000574	22	8 FEBRUARY 2000

FULL NAME(S) OF APPLICANT(S)

71	SOUTH AFRICAN SUGAR ASSOCIATION

FULL NAME(S) OF INVENTOR(S)

72	1. BOTHA; FREDERIK COENRAAD 2. GROENEWALD; JAN HENDRIK

TITLE OF INVENTION

54	THE REGULATION AND MANIPULATION OF SUCROSE CONTENT IN SUGARCANE

BACKGROUND OF THE INVENTION

THIS invention relates to the regulation and manipulation of sucrose content in sugarcane.

The amount of sucrose which accumulates in the culm of sugarcane is a function of sink strength and carbon partitioning between competing metabolic pathways.

Pyrophosphate-dependent phosphofructokinase (PFP) with the international identification of (EC 2.7.1.90) catalyses the reversible conversion of fructose 6-phosphate (F6P) and pyrophosphate (PPi) to fructose 1,6-bisphosphate and inorganic phosphate. As such, the enzyme probably plays an important role in regulating carbon partitioning, and therefore sucrose

levels, in sugarcane. The levels of both PPi and F6P control the rate of sucrose accumulation by affecting the rate of sucrose synthesis and hydrolysis. Consistent with this hypothesis, the activity of PFP in sugarcane increases with culm maturity, and varies between varieties differing in their sucrose storage capacity.

PFP is associated with sink tissues, and plays a major role in sink strength. PFP is a heterotetramer with two α and two β-subunits. The β-subunit is the catalytic subunit of the enzyme. Removal of the β-subunit will therefore reduce the PFP activity.

It is an object of the invention to use the sugarcane PFP-β gene or part thereof in an antisense and untranslatable form to regulate the levels of PFP in sugarcane.

SUMMARY OF THE INVENTION

According to the invention an isolated nucleotide sequence comprises:

(i) a nucleotide sequence as set out in Figure 1;

(ii) a nucleotide sequence which is complementary to the nucleotide sequence of (i);

(iii) a variant of the nucleotide sequence of (i);

(iv) a portion of the nucleotide sequence of (i); and

(v) a nucleotide sequence which hybridizes to the nucleotide sequence of (i) under stringent hybridization conditions.

The nucleotide sequence may be the nucleotide sequence as set out in Figure

3.

The nucleotide sequence may be in an antisense orientation.

According to another aspect of the invention a gene construct comprises a promoter and nucleotide sequence as defined herein in a sense orientation, the gene construct lacking a translation initiation codon upstream of the nucleotide sequence or possessing an in-frame termination codon directly downstream of the initiation codon.

The gene construct may comprise two promoters.

The promoters may be selected from the CaMV35S and the maize polyubiquitin (UBI) promoters.

According to another aspect of the invention a gene construct comprises a promoter and a nucleotide sequence as defined herein in an antisense orientation.

The gene construct may comprise two promoters.

The promoters may be selected from the CaMV35S and the maize polyubiquitin (UBI) promoters.

The gene constructs may be expression vectors, pUSPC 510 and pASPC 510 respectively.

According to another aspect of the invention a transformed sugarcane plant

cell comprises a gene construct of the invention.

According to another aspect of the invention a transgenic sugarcane plant or sugarcane plant part containing or derived from the plant cell is provided.

The transgenic sugarcane plant part may be a sugarcane callus.

The transformed sugarcane cell or transgenic plant or plant part may be characterized by a lower level of the PFP β-subunit.

The transformed sugarcane cell or transgenic plant or plant part may be characterized by a higher level of sucrose.

According to another aspect of the invention a method of regulating or manipulating the level of active PFP in a plant cell comprises the step of transforming the plant cell with at least one gene construct of the invention.

According to another aspect of the invention a method of maintaining or increasing the sucrose level in plant tissue comprises the step of transforming cells of the plant tissue with at least one gene construct of the invention.

According to another aspect of the invention a method of manipulating sucrose metabolism in a plant cell comprises the step of co-transforming the plant cell with each of the gene constructs of the invention.

The method may involve the alteration of sucrose metabolism in a plant or plant part containing stored sucrose.

The plant may be sugarcane.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention will now be described in more detail, by way of example only, with reference to the accompanying drawings in which:

Figure 1 is the nucleotide sequence of the sugarcane PFP-β gene;

Figure 2 is a flow diagram of the steps involved in the isolation and characterization of the sugarcane PFP-β cDNA fragment;

Figure 3 is the nucleotide sequence of the 1209 base pair (bp) cDNA fragment containing the 3' end of the PFP-β gene; and

Figure 4 is a schematic representation of the genetic constructs, pUSPC 510 and pASPC 510, containing the 1209 bp PFP-β cDNA fragment in an untranslatable and an antisense form, respectively.

DETAILED DESCRIPTION OF THE INVENTION

A sugarcane leafroll cDNA library for PFP-β cDNA was screened as described below. Fragments of the PFP-β cDNA were isolated from the cDNA library. As an example, the constructs of a set of expression vectors containing a 1209 BP fragment of the 3′-end of the sugarcane PFP-β gene

will be described in detail. The fragment was used to construct vectors which were used to transform cells in a sugarcane callus. One of the vectors, pUSPC 510, contained the fragment in a sense orientation but lacked a translation initiation codon. The other vector, pASPC 510 contained the fragment in an antisense form. It was found that the isolated gene fragments could be used to regulate or manipulate the level of active PFP in the cells thereby manipulating sucrose metabolism in the cells.

Referring to Figure 2, an amplified PFP-β cDNA fragment was used as a probe to screen a sugarcane leafroll cDNA library for the PFP-β cDNA. Sequencing analysis was done to characterize the isolated clone. The sequence is shown in Figure 3. The insert of the clone was removed and used in the construction of two plant expression vectors. A promoter cassette with the CaMV *35S* and the maize polyubiquitin (UBI) promoters was used in these constructs. In the first vector the fragment was cloned in the sense orientation, which is untranslatable because of the lack of a translation initiation codon. The fragment was cloned in the antisense orientation in the second vector. Schematic representations of the two expression vectors are shown in Figure 4.

Co-transformation with one of the expression vectors and a selectable marker gene of sugarcane callus was performed using a particle inflow gun and transformants were selected on geneticin-containing medium. Transformants were subsequently analyzed by measuring the PFP enzyme activity in the tissue with a standardised method (Botha et al. 1986). The PFP-β-fragment was excised from the library vector (pPFP 5) using the restriction enzymes Hinc III and Sma 1. After purification, this fragment was cloned into the Sma 1 site of the expression vector DUBI 920.

References:

Botha, F. C., Small J.G.C., de Vries, C. (1986) Isolation and Characterisation of pyrophosphate: D-fructose-6-phosphate 1-phosphotransferase from cucumber seeds. Plant Cell Physiology 27: 1285-1295.

The content of this document is incorporated herein by reference.

Dated this 8th day of February 2000.

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SPOOR AND FISHER
APPLICANT'S PATENT ATTORNEYS

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        10          20          30          40          50          60          70
ATGGCGGCGC CGAGCGGACC ATCACCTGGG ACTGGGAGGT TGGCGTCGGT TTACAGCGAG GTGCAGACGA
        80          90         100         110         120         130         140
GCCGCCTCCA TCACGCGATC CGGCTCCCCT CCGTCCTCTG CTCCCAATTC TCCCTCGTCG ATGGACCTCC
       150         160         170         180         190         200         210
CAGCTCAGCC ACGGGGAACC CGGATGAGAT CGCGAAGCTG TTCCCTAACT TGTTTGGGCA GCCGTCGGCG
       220         230         240         250         260         270         280
ACATTGGTGC CGGCCAAAGA GGCGGTGGAG GGGAAGGCGC TGAAGGTCGG GGTGGTGCTC TCTGGTGGAC
       290         300         310         320         330         340         350
AAGCACCCGG TGGGCACAAT GTGATCTGCG GTATCTTCGA TTTCTTGCAG AAACACGCAA AGGGAAGCAC
       360         370         380         390         400         410         420
AATGTATGGA TTCAAAGGAG GCCCAGCAGG GGTGATGAAG TGCAAGTACG TCAAACTCAA TACCGATTTC
       430         440         450         460         470         480         490
GTCTATCCCT ACAGAAACCA GGGTGGTTTT GATATGATCT GTAGTGGAAG GGATAAGATT GAAACACCAG
       500         510         520         530         540         550         560
AGCAGTTTAA GCAAGCCGAA GATACAGCCA ACAAACTTGA GTTGGACGGA CTTGTTGTTA TTGGACGGGA
       570         580         590         600         610         620         630
CGATTCAAAT ACTCATGCTT GCCTCTTTGC TGAATACTTC AGGAGTAAAA ATTTGAAAAC CCGTGTCATT
       640         650         660         670         680         690         700
GGCAGCCCAA AGACCATTGA TGGTGATCTC AAATGCAAAG AGGTTCCAAC CAGTTTTGGA TTTGACACTG
       710         720         730         740         750         760         770
CATGCAAGAT CTATTCAGAA ATGATTGGAA ATGTCATGAT TGATGCCCGA TCAACTGGAA AATATTATCA
       780         790         800         810         820         830         840
CTTTGTACGG CTTATGGGGC GTGCTGCTTC TCACATTACA TTGGGATGCG CTTTGCAAAC ACACCCCAAT
       850         860         870         880         890         900         910
GCTGCACTCA TTGGGGAAGA GGTTGCTGCA,AAGAAGCAAA CCCTTAAGAA CGTCACAAAC TACATTACTG
       920         930         940         950         960         970         980
ATATCATCTG CGAGCGTGCA GATCTTGGTT ACAACTATGG TGTTATCCTT ATACCAGAAG GCCTGATTGA
       990        1000        1010        1020        1030        1040        1050
TTTCATCCCA GAGGTGCAGA ATATCATTGC TGAATTGAAT GAAATTTTGG CACATGATGT TGTTGATGAG
      1060        1070        1080        1090        1100        1110        1120
GCAGGGGCCT GGAAAAGCAA GCTTCAGCCT GAATCAAAGG AGCTGTTTGA GTTTTTGCCC AAAACTATTC
      1130        1140        1150        1160        1170        1180        1190
AGGAGCAACT TATGCTTGAA AGGGGCCCCC ATGGCAATGT TCAGGTTGCA AAAATTGAAA CCGAGAAAAT
      1200        1210        1220        1230        1240        1250        1260
GCTTATTAGC ATGGTGGAAA CTGAACTGGA GAAGAGAAAA GCAGAGGGGA GATACTCTGC ACATTTCAGA
      1270        1280        1290        1300        1310        1320        1330
GGGCAAGCTC ATTTCTTTGG GTACGAAGGA AGATGTGGCC TTCCTACCAA TTTTGATTCT AACTATTGCT
      1340        1350        1360        1370        1380        1390        1400
ATGCATTAGG CTATGGGGCT GGTGCCCTTC TCCAAAGTGG GAAGACAGGA CTTATTTCAT CGGTTGGCAA
      1410        1420        1430        1440        1450        1460        1470
CCTTGCGGCT CCAGTAGAAG AATGGACTGT TGGTGGAACA GCATTGACAT CACTGATGGA TGTGGAGAGG
      1480        1490        1500        1510        1520        1530        1540
AGGCATGGCA AGTTCAAGCC AGTGATCGAG AAGGCTATGG TGGAACTTGA TGCTGCACCT TTCAAGAAAT
      1550        1560        1570        1580        1590        1600        1610
ATGCATCAAT GCGGGATGAG TGGGCCACCA AGAACAGATA CATCAGCCCT GGCCCCATCC AGTTCAGTGG
      1620        1630        1640        1650        1660        1670        1680
CCCTGGAAGT GATGACTCGA ACCACACTTT GATGCTGGAA CTCGGTGCTG AGTTATAG.. ..........
```

Figure 1. The nucleotide sequence of the sugarcane PFP-β gene.

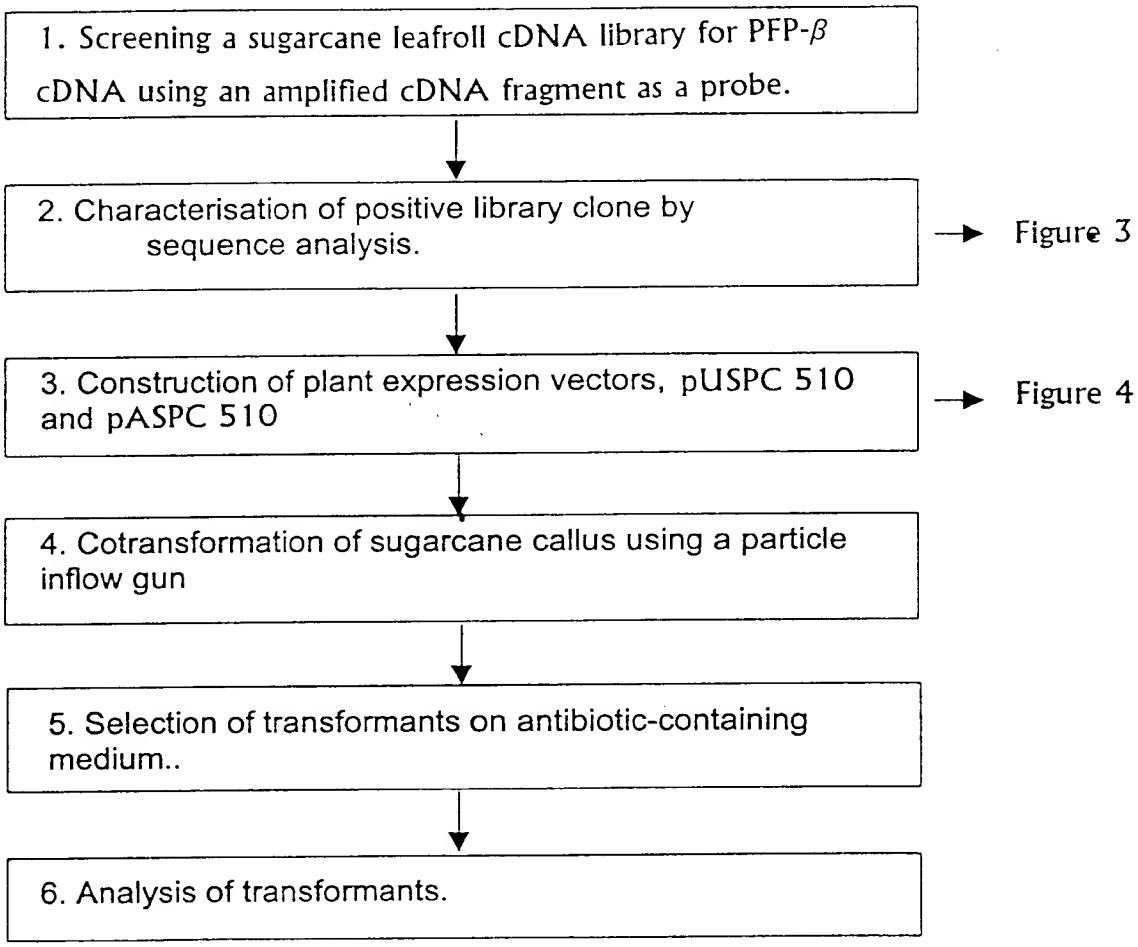

Figure 2. Flow diagram of the steps involved in the isolation and characterisation of sugarcane PFP-β cDNA and construction of expression vectors for the manipulation of sucrose metabolism in sugarcane.

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        10          20          30          40          50          60          70
GACGGTATCG ATAAGCTTGA TATCGAATTC CGATTTAGCC TCATACTGCT TCTCACATTA CATTGGGATG
        80          90         100         110         120         130         140
CGCTTTGCAA ACACACCCCA ATGCTGCACT CATTGGGGAA GAGGTTGCTG CGAAGAAGCA AACCCTTAAG
       150         160         170         180         190         200         210
AACGTCACAA ACTACATTAC TGATATCATC TGCAAGCGTG CAGATCTTGG TTACAACTAT GGGGTTATCC
       220         230         240         250         260         270         280
TTATACCAGA AGGCCTGATT GATTTCATCC CAGAGGTTCA AAAACTCATC GCAGAATTGA ATGAAATTTT
       290         300         310         320         330         340         350
GGCACATGAT GTGGTTGATG AGGCAGGGGC CTGGAAAAGC AAGCTTCAGC CTGAATCAAA GGAGCTGTTT
       360         370         380         390         400         410         420
GAGTTTTTGC CCAAAACTAT TCAGGAGCAA CTTATGCTTG AAAGGGGCCC CCATGGCAAT GTTCAGGTTG
       430         440         450         460         470         480         490
CAAAAATTGA AACCGAGAAA ATGCTTATTA GCATGGTGGA AACTGAACTG GAGAAGAGAA AAGCAGAGGG
       500         510         520         530         540         550         560
GAGATACTCT GCACATTTCA GAGGGCAAGC TCATTTCTTT GGGTACGAAG GAAGATGTGG CCTTCCTACC
       570         580         590         600         610         620         630
AATTTTGATT CTAACTATTG CTATGCATTA GGCTATGGTG CTGGTGCCCT TCTCCAAAGT GGGAAGACAG
       640         650         660         670         680         690         700
GACTTATTTC ATCGGTTGGC AACCTTGCGG CTCCAGTAGA AGAATGGACT GTTGGTGGAA CAGCATTGAC
       710         720         730         740         750         760         770
ATCACTGATG GATGTTGAGA GGAGGCATGG CAAGTTCAAG CCAGTGATCA AGAAGGCTAT GGTGGAACTT
       780         790         800         810         820         830         840
GATGCTGCAC CTTTCAAGAA ATATGCATCA ATGCGGGATG AGTGGGCCAC CAAGAACAGA TACATCAGCC
       850         860         870         880         890         900         910
CTGGCCCCAT CCAGTTCAGT GGCCCTGGAA GTGATGACTC GAACCACACT TTGATGCTGG AACTCGGTGC
       920         930         940         950         960         970         980
TGAGTTATAG AGATGCGTCC TTTGCTTATT TTTGTTTCTT ACAGTTTTGG GAGTGGAGAC TGGACACTGG
       990        1000        1010        1020        1030        1040        1050
GTCTCCTGGA GCAGCCTGCA GTCTCCATAT TGTGAATTGT TTAATAAGAG GTTCGATGTG AGTTTTCTGC
      1060        1070        1080        1090        1100        1110        1120
GTAGCGGACT GGATGTAGCA AATAAGAACT GGTTTTAGCA TTTTTTGTAT GATTTACGCA CCAACTGACT
      1130        1140        1150        1160        1170        1180        1190
TGTCTTGTAA CCCTGATTCT GTTCCACTGG TTGCAATCTC GTGAGAATGA ACAAGTTGAT ATGAGGCTAA
      1200        1210        1220        1230        1240        1250        1260
ATCGGAATTC CTGCAGCCC. .......... .......... .......... .......... ..........
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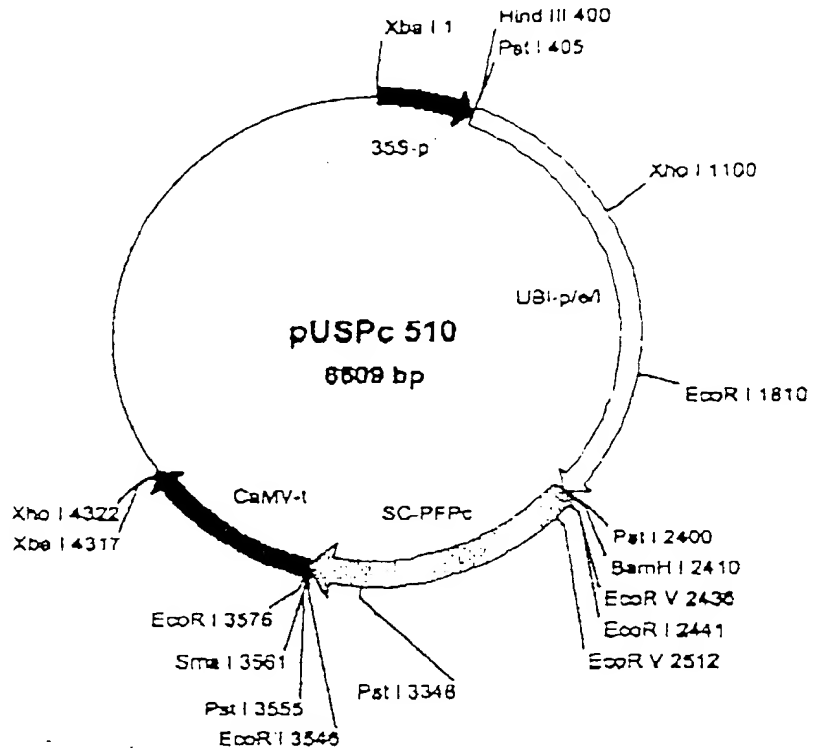
Figure 3. Nucleotide sequence of the 1209 bp fragment of the 3′-end of the sugarcane PFP-β-gene isolated from a cDNA library. The termination codon (TAG) is underlined (bp 918).



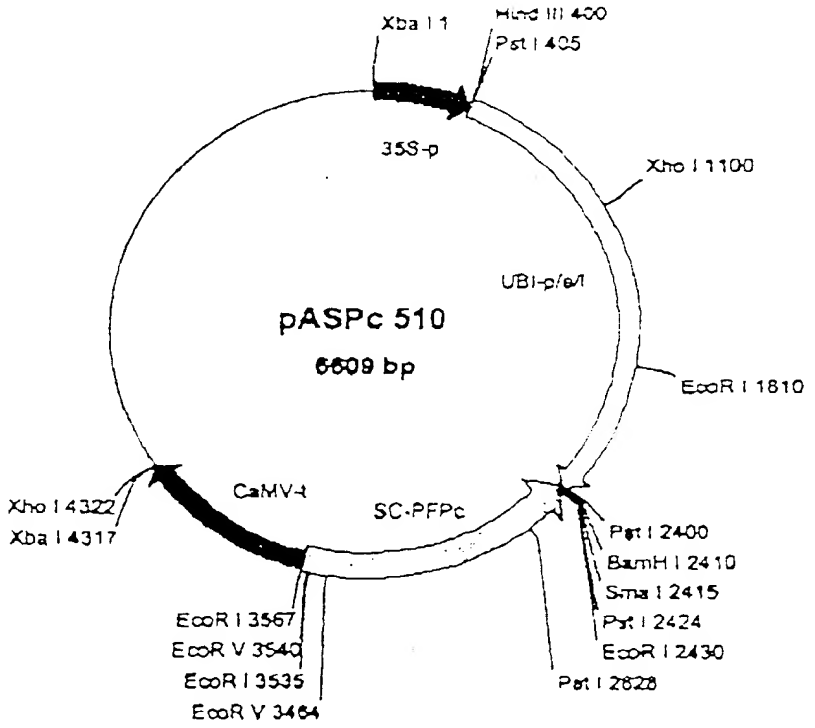


Figure 4. Schematic representation of the genetic constructs, a.) pUSPC 510 and b.) pASPC 510, containing 1209 bp of the PFP-β cDNA in the untranslatable (U) and antisense (A) forms respectively as examples of expression vectors containing sugarcane PFP-β sequences.